PRODUCTION OF INTERLEUKIN-6 OVER A HYPERTHERMIC RANGE BY LIPOPOLYSACCHARIDE STIMULATED NEUTROPHILS

by

Daniel J. Bessmer

A Thesis Submitted in

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The University of Wisconsin-Milwaukee, 1999

Under the Supervision of Dr. John Ndon

Neutrophils are an essential cell of the immune system, responsible for phagocytosis of bacterial pathogens. However, their role in immunity is not limited to this action. The neutrophil response to and production of cytokines is paramount to successful immune responses. Interleukin-6 (IL-6) is an important cytokine produced by the neutrophil. This cytokine has pleotropic activity but of interest in this study is its ability to act as an endogenous pyrogen and cause fever. It was wondered what the effect of elevated temperature would have on the neutrophil production of IL-6. It was hypothesized that elevated temperature would decreased the amount of neutrophil produced IL-6. Neutrophils were isolated by density gradient centrifugation then standardized to 10^6 per mililiter in cell culture fluid. The cells were primed with fMLP and subsequently stimulated with the bacterial endotoxin, lipopolysaccharide (LPS) serotype O111:B4. To mimic the conditions of fever, the cells were incubated at various temperatures representing a range from normal to severe fever. Samples of culture supernatant were

pulled at various time intervals and later assayed for IL-6 levels. The results reveal that LPS stimulated neutrophils produced picogram quantities of IL-6. The results among three batches were highly variable but noticeable trends were present. On average, normal body temperature (37° C) levels of IL-6 were more than either 38.5° C or 40°C. However at 41° C, levels of IL-6 were much higher than base line. This suggests that during mild and moderate fever IL-6 levels may be slightly depressed, but are enhanced at the upper end of the physiological range. Other studies are needed to show how the mechanism by which this apparent rise in IL-6 is controlled.

Major Professor

Date

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Chapter 1

INTRODUCTION

Neutrophils are popularly known for their phagocytic functions. However, their role in immunity is not limited to seeking out and engulfing pathogenic bacteria. Neutrophils produce a number of proteins that have a wide range of immune mediating functions.[1] The cytokine interleukin-6 (IL-6), is one such molecule produced by the neutrophil. Macrophages are more widely known to produce this cytokine, but neutrophils also produce IL-6 in response to varied stimuli. Although macrophages produce about 10-fold more IL-6 on a per cell basis, neutrophils are much more numerous during initial stages of infection, and are often more than 10 times the amount of monocytes in peripheral blood, therefore they contribute significantly to the total production of IL-6.[2]

Interleukin-6 is a cytokine with pleotropic action. In normal immune responses it stimulates the liver to produce acute phase proteins, and also promotes differentiation of B-cells and T-cells.[3,4] It synergistically acts with IL-3 to regulate normal hematopoesis. In pathophysiologic conditions it has been implicated to play a role in autoimmune diseases, osteoporosis, neoplasms and sepsis.[3] Sepsis can often be caused by infection with gram negative bacteria. During the infection and subsequent host reaction, increased levels of the lipopolysaccharide enodtoxin is released from the bacterial cell membrane and stimulates IL-6 production. IL-6 is a potent endogenous pyrogen. It can quickly produce fever when injected in various

animal models and humans.[5] IL-6 affects the thermoregulatory center in the hypothalamus and thereby produces changes in body temperature.[6] This effect prompted the current investigation to associate the effects of elevated temperature on IL-6 production by neutrophils. The specific aims were:

1. To measure IL-6 protein levels in cell culture supernatant.
2. To measure IL-6 expression as a function of time and LPS stimulation.
3. To correlate the effects of increased temperature on the regulation of IL-6 expression.

It was hypothesized that elevated temperature would be a negative feedback signal to decrease the production of the cytokine.

Chapter 2

LITERATURE SEARCH

A. Neutrophils

Neutrophils are well known for their phagocytic functions, but their contribution to immunity as a whole goes beyond that. They possess complex systems that allow cell-to-cell interactions among other leukocytes as well as the ability to affect other tissues throughout the body. Neutrophils are derived from a myeloid progenitor cell in the bone marrow. They comprise 55-60% of the nucleated cell population in the marrow at various stages of development.[7] The mature neutrophil lives in the circulation for 8-20 hours, then marginates to the vasculature and tissues where it may survive for several days.[2] In the early 1970s McCall [8] was among the first to show that neutrophils were not terminally differentiated by describing changes in oxidative metabolism, phagocytosis, chemotaxis and 2-deoxyglucose transport in what was termed "toxic" neutrophils. During this time work was being done with formylated oligopeptides [7] that demonstrated that these peptides could stimulate an upregulation of function of mature neutrophils, although at low concentrations they did not fully activate the cell. This partial stimulation, called priming, lends the cells to enhanced respiratory burst, greater phagocytosis, and higher levels of degranulation over unprimed neutrophils. With improved molecular biology techniques in the 1980s, many of the cytokine genes were cloned and characterized.[9-14] It

was possible to obtain large quantities of purified cytokines to study their effects on cell physiology. Berton and colleagues showed that γ-interferon could prime neutrophils for reactive oxidant release.[15] This effect was abolished with incubation with cycloheximide, proving that the response after priming was dependent on new protein synthesis. Studies during this time showed that neutrophils could be stimulated by cytokines, and they also produced cytokines and other molecules that modulated proinflamatory changes in a variety of cell types.[16-23] It became clear therefore, that neutrophils did in fact enhance immune reactions by responding to or secreting newly formed biochemicals, and were not relegated to simple phagocytosis.

B. Lipopolysaccharide

Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of gram negative bacteria. The endotoxin must be released by bacterial degradation before its toxicity is exerted. LPS is composed of three subunits: The O antigen, the core antigen, and lipid A.[24] The O antigen is a polysaccharide chain and is the most variable portion in terms of its structure. The Core antigen is comprised of sugar moieties and ketodeoxyoctanoic acids. Finally, the Lipid A portion is made of sugars, phosphate groups, ketodeoxyoctanoic acid, and fatty acids. The fatty acids allow it to stably integrate into the bacterial membrane. Lipid A is the toxic portion of the molecule.[24,25] LPS-mediated toxicity is related to its ability to activate complement and induce cytokine production.[24,26] Cytokines are responsible for the ensuing inflammatory reaction. They stimulate formation of products such as

prostaglandins, leukotrienes, and also fever induction.[24,27] Normally the inflammatory reaction is confined to a focus of infection. However, when an LPS-induced reaction spreads to the circulation it becomes systemic, sepsis results and causes significant morbidity and mortality, owing in part to the effects of cytokines.[29] It takes low ng quantities of endotoxin to induce the complications of sepsis which include respiratory distress syndrome, disseminated intravascular coagulation and multi-organ dysfunction syndrome.[28] LPS mediates its effects by binding to a specific receptor, CD14.[29] CD14 is a 55Kd glycoprotein that is glycosylphosphatidylinositol (GPI) anchored to the membrane of neutrophils and monocytes.[30] LPS binding to CD14 is aided by another molecule called lipopolysaccharide binding protein (LBP.) LBP is a 60kd glycoprotein that catalytically transfers LPS to CD14, and shares homology with other lipid transfer proteins. [25,30,31] A putative binding site on LBP for LPS has been elucidated by site directed mutagenesis and is necessary for binding, transfer, and stimulation of CD14 bearing cells.[31] Lipid A is the portion of LPS that binds LBP in a one-to-one stoichiometry.[31] Experiments show that LPS binding to CD14 is increased $10^2 - 10^3$ times in the presence of LBP.[32,33] Neutrophil functions have been shown to be enhanced in the presence of purified LBP and with serum or plasma,[34,35] whereas hyporesponsiveness is seen with washed neutrophils. This demonstrates the need for addition of LBP to the test system when neutrophils are isolated in a plasma free environment and LPS will be used to stimulate the cells.

C. INTERLEUKIN-6

Interleukin-6 (IL-6) is a glycoprotein cytokine. It is known by many synonyms: β_2-Interferon, B-cell Stimulatory Factor, Hepatocyte Stimulating Factor, Hybridoma/Plasmacytoma Growth Factor, Monocyte Granulocyte Inducer type-2, T-cell differentiation Factor, and Thrombopoietin.[3,36] This list attests to its wide range of functions.

(i) Gene Expression

The gene for IL-6 is located on chromosome 7p21 and is approximately 5Kb. The gene was cloned in 1986 [37] and since has been extensively studied. *IL-6* expression is controlled both positively and negatively by a number of factors. In its promotor region there are three transcription initiation sites, each with a TATA-like sequence.[38] Transcription is promoted by various stimuli. The promotor has a multiple response element and a Nuclear Factor-IL6 (NF-IL6) response element embedded within a serum response element, and a binding site for Nuclear Factor kappaB (NFkappaB).[36] NF-IL6 is activated by a Ras dependent cascade [39] induced by (but not limited to) IL-1_β, TNF_α and LPS.[3] Molecular deletion studies with NFkappaB show that it is directly involved with *IL-6* gene induction.[40,41] Signal transduction studies with CD14 show that NFkappaB is mobilized from a cytoplasmic bound form by the action of protein tyrosine kinases.[42,43] Repression of *IL-6* transcription occurs with glucocorticoids, estrogens, androgens, c-fos, and wildtype and mutant p53.[36] Glucocorticoids are often elevated during inflammation to maintain a careful balance

between proinflamatory and antiinflamatory processes. The IL-6 promotor has a response element for glucocorticoids that serves to inhibit NFkappaB binding.[36] Similar mechanisms exist for androgen and estrogen repression of *IL-6* transcription.[44] The importance of this hormonal repression mechanism is underscored by the observation of osteoporosis development following ovariectomy. This pathophysiologic process occurs as a result of increased IL-6 levels which stimulate osteoclastogenesis, resulting in bone loss.[45] In the neutrophil, IL-6 mRNA is not constituitively expressed in neutrophils and therefore requires stimulation to produce the protein.[46]

(ii) IL-6 Protein

This protein has a molecular weight range of 21-28Kd depending on the degree of phosphorylation and glycoslyation.[47] The mature peptide is 184 aa. after cleavage of a 28 aa. signal peptide. Human IL-6 shares 42% homology with murine IL-6, however, there is a centrally conserved region with 57% homology containing four perfectly aligned cystine residues.[48] This protein belongs to the IL-6-type family of cytokines, which are mainly involved in cell growth and differentiation. IL-6 is produced by many cell types and acts to enhance specific and innate immune reactions. The cytokine causes differentiation and immunoglobulin production of activated, but not resting, B-cells.[49] Along with IL-1 it induces differentiation of T-cells to cytolytic T-cells and activates natural killer cells.[50,51,52] IL-6 participates in the

acute phase response during inflamation by stimulating hepatocytes to produce acute phase proteins.[53] Additionally, this cytokine stimulates hematopoiesis, regulates bone metabolism, serves as a growth factor for myeloma and other neoplasias, and plays a role in autoimmune disease.[2,36,53] IL-6 also acts as an endogenous pyrogen.[54] The effects of this cytokine all require specific ligand binding to the IL-6 receptor.

(iii) IL-6 Receptor

The receptor for IL-6 (CD126) is comprised of two polypeptide subunits. The first is gp80, the actual binding site for IL-6, and is an 80Kd protein belonging to the immunoglobulin superfamily.[53] The intracellular region does not possess any kinase domains, suggesting the necessity for another molecule capable of signal transduction.[55] That molecule is gp130, which is a signal transducer with Janus Kinase (Jak) and Signal Transducer and Activator of Transcription 3 (STAT-3) activity.[56] Other cytokines in the IL-6-type family also use gp130 as their signal transducer.[53] Binding to the gp80 subunit forms a low affinity complex, which then triggers association with gp130 to form a higher affinity complex.[36] Actually, the high affinity complex is hexameric with two molecules each of IL-6, gp80, and gp130.[57] The gp80 subunit is also found as a soluble component in serum and other body fluids.[58] The soluble receptor, likewise, forms a hexameric complex with membrane bound gp130, leading to subsequent signal transduction.[57]

(iv) IL-6 Induced Fever

The pyrogenic activity of IL-6 has been well described.[59,60,61] The induction of fever is related to the ability of IL-6 to affect the hypothalamous.[6] Cold sensitive neurons, as opposed to their counterpart warm sensitive neurons, are selectively stimulated by IL-6 resulting in a resetting of the thermoregulatory center.[61] The brain interprets the signal as a need to raise body temperature. The neuroimmune repsonse of the nervous system, both the peripheral and central, has been shown to be modulated by the neurotropic effects of IL-6.[64,65] Experiments with IL-6 deficient mice were key to the understanding some of the neurochemical changes associated with the febrile response.[66] The mechanism to induce fever is believed to be mediated by generation of prostaglandin E2 after exposure to the cytokine.[62,63] In another study it was shown that severing the subdiaphragmatic vagal nerves abrogated the febrile response compared to sham operated animals, from intraperitoneal LPS injections.[67] This demonstrates a communication pathway for the immune response to signal the brain to induce fever. Furthermore, IL-6 activity in plasma after LPS injection correlates to the febrile change in body temperature.[68] A direct correlation was also found for IL-6 levels in hypothalamic push-pull perfusates and body temperature after LPS injection.[69] Therefore, it seems clear, that there is a direct causal relationship between LPS, IL-6 and fever development, which is why this study was undertaken.

Chapter 3

MATERIALS AND METHODS

Materials

Histopaque™ 1.007 (#1.077-1), 1.119 (#1.119-1), fMLP (#F3506) lipopolysaccharide O111:B4 (#L4130) and RPMI-1640 with glutamine (#R8758) were purchased from Sigma Chemical Company (St.Louis, MO). The ELISA kit for IL-6 (Quantikine® #D6050) was purchased from R&D Systems (Minneapolis, MN). All reagents and stock solutions were prepared with endotoxin-free, cell-culture grade water (W3500, Sigma).

Isolation of Neutrophils

Whole blood, from a single healthy donor, was drawn into sterile 5 ml lithium heparin tubes (Becton-Dickenson). Three mls of histopaque 1.119 were placed in the bottom of a sterile 15 ml conical polystyrene centrifuge tube. On top of this, 3 mls of histopaque 1.077 were carefully layered. Whole blood was then carefully layered on top of the histopaque 1.077. The tubes (four sets) were then centrifuged at 700g for 30 minutes. After centrifugation an aliquot of plasma was removed with a sterile transfer pipette and placed in a sterile 15 ml conical tube. The remaining plasma was carefully aspirated with a sterile transfer pipette and discarded. The mononuclear cells were aspirated and discarded along with most of the 1.077 solution, stopping just shy of the 1.077/1.119 interface. The granulocytes were then harvested with a

sterile transfer pipette and placed into a single sterile 15 ml conical centrifuge tube. RPMI-1640 was added to the 10ml level and the cells were centrifuged for 10 minutes at 200g. The supernatant was removed by a sterile transfer pipette and discarded. At this point the cells were gently resuspended in the remaining RPMI (at the 1 ml graduation) and exposed to 0.2 % (wt/vol) saline to lyse any contaminating red blood cells. RPMI was again added to the 10ml mark and the cells washed as above for a total of 3 times.

Standardizing the Neutrophils

After washing, the cells remained in approximately, but not less than, 1 ml. A small aliquot was removed and mixed with an equal volume of trypane blue. This mixture was then placed into a standard hemacytometer and the cells were enumerated by bright field microscopy. All cells were counted and a separate tally of nonviable cells was kept. The total number of cells per ml was calculated. The percent of viable cells was also calculated and used to correct the total count to viable cells per ml. All cell separations done throughout the experiment were at least 95% viable. The number of viable cells was then used to calculate the volume of cell suspension and RPMI-1640 needed to bring the count to 1.0×10^6 cells per ml. Once the cells were standardized, autologous plasma was added at 1.0 percent (vol/vol) to supply endogenous lipopolysaccharide binding protein (LBP). To assess the purity of the cell preparation, one drop of cell suspension was added to one drop of plasma and saline. This mixture was spread on a glass slide and air dried. The slide was stained

with a wright's stain and examined on a microscope for the cell types present. All preparations were at least 98 % neutrophils.

Exposure of the Neutrophils

An aliquot (200µl) was removed from the cell suspension to serve as a negative control of native cells. The remaining cells were aliquoted into four tubes. N-formyl Met-Leu-Phe was then added to each tube at a final concentration of 5 ng/ml, an amount that has been demonstrated to be adequate for priming. One tube received no other stimulation and served as a "primed only" control. After 15 minutes of priming, the other tubes received LPS at a final concentration of 1ng/ml, 10 ng/ml, and 100 ng/ml respectively. This general range was also known to be adequate for stimulation, but it was not known if elevated temperature would diminish stimulation. Therefore, multiple concentrations were selected to make certain to find any dose dependent differences in IL-6 production. The cells were then incubated in 5% CO_2 for 2 hours at 37° C. 200µl was removed from each LPS exposed tube and centrifuged at 3000 RPM for 3 minutes to pellet the cells. The supernatant was then collected and frozen at -70° C. This was repeated at 4, 6, and 8 hour intervals. Since it was not known when IL-6 would be detected, time intervals were used to catch any temperature dependent differences in cytokine production rather than just one time. After 8 hours the negative control tubes were centrifuged and the supernatant was collected and frozen. An additional viability check was performed after eight hours on the remaining cells from selected tubes. The entire procedure was performed

again for incubations at 38.5°C, 40°C, and 41°C. The temperature was verified by a thermometer placed in the incubator. To check the thermal stability of IL-6, an aliquot of recombinant human IL-6 (100pg/ml) was incubated at 41°C for 8 hours and then frozen at -70°C until assayed.

IL-6 Assay

The assay for IL-6 was obtained from R&D Systems, Quantikine human IL-6, and is a standard sandwich enzyme-linked immunosorbant assay. The sensitivity was 0.7 pg/ml and had standards ranging from 0.0 to 300 pg/ml. The manufacturers instructions were followed. A monoclonal antibody specific for IL-6 was precoated in a 96 well microtiter plate. After 100ul sample addition, the plate was incubated for 2 hours at room temperature. The wells were washed 4 times with the supplied wash buffer. 200µl of anti-IL-6 polyclonal antibody, conjugated to horseradish peroxidase, was added to each test well. The plate was incubated for another two hours at room temperature. The wells were again washed as above, and followed by addition of 200µl of the substrate solution, tetramethylbenzidine and hydrogen peroxide. After 20 minutes the reaction was stopped by adding 50 µl of 2N sulfuric acid. The absorbance of the plate wells was determined by a plate reader (BioRad model 3550) at 450nm with wavelength correction at 670nm. The concentration of each sample was calculated based on a standard curve of recombinant human IL-6 supplied in the

kit. The absorbance readings were processed using Biorad Microplate Manager software version 2.0.3.

Chapter 4

RESULTS

As expected, in all cases the unstimulated native cells and the fMLP primed only cells did not produce any detectable IL-6. Additionally, all cell preparations were >98% neutrophils with only rare eosinophils and exceptionally rare nonidentifiable cells (due to cytoplasmic or nuclear condensation) being noted. Viability of the cells, as measured by trypane blue, were always above 95% both before and after 8 hours incubation. To check the thermal stability of IL-6, 100 pg/ml of recombinant human IL-6 was assayed after 8 hours of incubation at 41°C and compared to 100 pg/ml processed at room temperature. No difference was noted in the recovery of IL-6 between the two samples (Figure 1.)

The IL-6 assays were run on triplicate batches, each on a different day. The results were highly variable among each batch and the amounts and patterns of detection were not reproducible. Table 1 shows the IL-6 levels of each batch grouped by temperature and amount of LPS used to stimulate the cells. The first batch showed the greatest response in terms of amount of IL-6. The base line (37°C) levels with 1ng/ml LPS were not detected until 4 hours and continued to increase at 6 and 8 hours and were 0.9, 7.7, and 9.7 pg/ml respectively. A similar pattern was seen with 10 and 100 ng/ml of LPS as well. At 38.5° C IL-6 was decreased in amount and not detectable until 6 hours with 1 and 10ng/ml LPS, but not detectable until 8 hours when using 100 ng/ml LPS. At 40° C IL-6 was below base line levels as well, but

more than that at 38.5°. IL-6 was detectable by 4 hours and continued to increase at 6 and 8 hours. However, at 41° C the largest amount of IL-6 was produced. At 10 ng/ml LPS it was detectable at 2 hours (1pg/ml), otherwise it was detectable at 4 hours with 1 and 100 ng/ml LPS. The amounts detected at 4 hours were greater than base line levels at 8 hours. The amounts at 8 hours were 49.2, 41.8 and 42.4 pg/ml respectively for 1, 10, and 100 ng/ml LPS stimulation. Figure 2 shows batch 1 results by temperature and LPS amount used.

The second assay gave far different results. Base line levels were not detectable at all for 1 and 10 ng/ml LPS. Only 100 ng/ml LPS showed results at 6 and 8 hours of 1.2 and 1.0 pg/ml respectively. At 38.5° C again the only results were with 100ng/ml LPS but were detectable at 4, 6 and 8 hours. There was a slight dip at 6 hours (Fig3.) At 40° C only 100 ng/ml showed results at 4 hours and was not detected at 6 or 8 hours. The IL-6 detected at 41° C was the highest for this batch. Here it was detectable with 1, 10 and 100 ng/ml LPS. Although not detected until 8 hours with 1 ng/ml LPS, it was detected at 4 hours with 10 ng/ml LPS and at 2 hours with 100 ng/ml LPS. The levels peaked at 6 hours and fell slightly at 8 hours (Fig 3.)

Batch 3 additionally gave results of a different pattern(Fig 4). IL-6 was detected at base line only after 8 hours and was 2.5, 2.8, and 1.4 pg/ml for 1, 10, and 100 ng/ml LPS respectively. At 38.5° C the IL-6 was detected at 8 hours with 1 and 100 ng/ml LPS at 2.7 and 1.5 pg/ml but not detected at all for 10 ng/ml LPS. At 40° C no IL-6 was detected for any level of LPS. However, at 41° C the highest level of IL-6 was

again detected. Here at 1 ng/ml LPS 1.5 and 2.1 pg/ml IL-6 were found at 6 and 8 hours respectively. At 10 ng/ml LPS, it was detected at 6 and 8 hour as 1.4 and 4.7 pg/ml respectively. This 8 hour sample was the highest IL-6 level in the batch. Finally at 100 ng/ml LPS, IL-6 was found only at 8 hours and was 2.1 ng/ml.

Chapter 5

DISCUSSION

Cytokine production by leukocytes is an integral process in normal immune function. Interleukin-6 is a cytokine produced by many cells, including the neutrophil, resulting in the up-regulation of various cellular processes, such as acute phase protein production by hepatocytes, B-cell differentiation and immunoglobulin secretion, and induction of the fever response. Conversely, normal neutrophil function has been shown to depend on the presence of IL-6.[70] In this study IL-6 deficient mice did not mount a neutrophilic response to *E. coli* and lead to an accumulation of viable bacteria in tissue. Treatment with recombinant IL-6 resulted in restoration of a neutrophilia, decreased accumulation of bacteria, and increased survival. IL-6 can also be involved with a myriad of pathophysiological processes when its regulation becomes unbalanced. For example, multiple myeloma, a plasma cell dyscrasia is often associated with increased levels of IL-6 production and furthermore can be a marker for prognosis.[71] It is also associated with autoimmune disease, osteoporosis, and other neoplasms.[3]

Lipopolysaccharide, derived from gram negative bacteria, plays an important role in the pathogenesis of endotoxemia. Its presence will induce the formation of cytokines during the inflammatory response. This process is dependent upon cellular binding of LPS to its receptor, mainly on phagocytes. This is aided by an acute phase protein,

LBP, normally found in small amounts in plasma. The ensuing cascade of events may lead to a systemic reaction known as sepsis. During sepsis, cytokine levels rise and exert their effects by eliciting formation of inflammatory mediators which produce the pathophysiological state. Fever is a hallmark of sepsis and can be directly associated with pyrogenic cytokine levels. IL-6 has been shown not only to promulgate the febrile reaction but also correlate with survival rates during sepsis.[72,73]

The association between IL-6 as a mediator of endotoxemia, and neutrophil response to LPS and production of IL-6 has compelled this investigation. No studies have comprehensively shown the correlation of neutrophil IL-6 production in response to LPS over a hyperthermic range which represents mild, moderate, and severe fever. By revealing the effects of elevated temperature on the production of IL-6, it may eventually aid intervention strategies for treatment of fever and sepsis.

The results of these experiments show a high degree of variability among batches. When looked at in the aggregate though, some trends appear. First, detectable IL-6 usually appeared at either 4 or 6 hours. This is consistent with other reports.[6,7] When compared to base line levels at 37° C, the production of IL-6 at 38.5° C and 40° C was lower, implying that for these temperatures there is a decreased production. Suprisingly though, was the fact the in all cases the highest production of IL-6 occurred at 41°C. Not only was it higher, but it was detected earlier. It was the only temperature where the cytokine was detected at 2 hours time. This effect may be due

to temperature dependent factors that effect transcription, posttranscriptional modification, translation, or posttranslational modification events.

LPS dose dependency was not clearly demonstrated because there were instances that showed it, and others that did not. This would be related to the available CD14 receptors on the neutrophils and once saturated any excess would not effect results. There are only about 3000 CD14 molecules per neutrophil [74] which makes it easy to saturate with ng amounts of LPS. Stimulation with LPS has been shown to require a source of LBP. Many studies clearly show a significant difference in LPS stimulated cell activity, with and without, serum or plasma. A typical study of the kinetics for neutrophil activity showed that 1/2 maximal activity was achieved by a concentration of 0.13% plasma.[35] Volumes of 1% are adequate to achieve full stimulation by LPS.[34] In this current study, LBP was supplied to the test system by adding 1% autologous plasma to the cell suspension.

The variability of results may stem from biological or technical factors. Certainly, one confounding biological factor is that each day of cell separation, an aliquot of plasma was used from that particular blood draw. Therefore, identical conditions may not have been replicated in terms of the content of the plasma. There may have been differing amounts of LBP each time. Additionally, other unidentified components may have influenced production of IL-6, either positively or negatively. It is known that some hormones play a negative regulatory role in IL-6 production and the blood

samples were drawn without regard to the natural fluctuation of hormones in this female blood donor. Neutrophils are effected by estrogen via a receptor based system at concentrations as low as 10^{-10}M.[75] At 1% of its original concentration, it is difficult to attribute any repressive effects of hormones, however the cells may have been under the influence of natural amounts hormones at the time of the blood draw. This variable could have been removed by using purified LBP or the same batch of plasma for all cell exposures. It is important to point out that these results represent the effects seen in only one donor. This removes any person-to-person bias but does limit interpretation because not all people would produce the same pattern. It would be prudent to examine the effect of pooled neutrophils of multiple donors to see if the effect is the same.

Although there was a definite pattern among the results insofar as relative amounts of IL-6 at different temperatures, there was poor reproducibility among the three separate assays. Technical factors may account for this. Every attempt was made to perform each run the same however, significant differences of results did occur. Possible limitations can be attributed to several factors. First, there were three separate assays on three separate days. Each assay had its own standard curve generated and there may have been differences in the variables that would affect test performance. However, this was probably not the case because the standard curves were excellent. They were linear, had intercepts near zero and the absorbance values for a given standard amount was nearly the same for the different assays. Even

though incubation temperatures were verified by an internally placed thermometer, the fluctuations in temperature experienced by each of the twelve rounds of cell separations, would be different. Another factor for the cell responses, or lack of, may have been attributed to degradation of the fMPL priming agent or LPS which may have lost potency over time. Eight weeks elapsed between reagent preparation and last usage. This could account for ineffectual priming or stimulation of the cells. One experiment (figure 5) demonstrated that indeed the older reagents did not stimulate as much IL-6 as when both were new, and shows that some degradation did occur over time. Therefore the third batch of experiments used newly prepared fMLP and LPS stock solutions and may, somewhat, account for different results in this batch. The cell suspensions were prepared based on data from cell counts done on a counting chamber. This technique is highly imprecise compared to electronic cell counting. Therefore, the cell suspensions would have had variable cell concentrations on different days. Also human factors such as non-reproducible pipetting or other handling techniques may account for some of the differences. The possibility exists that during isolation of neutrophils the cells can become activated. Previous studies comparing whole blood and isolated neutrophils, which included RBC lysis, showed differences in CD11b, CD18, and CD16 antigen expression but not of CD14.[74, 76, 77] Therefore the stress from hypotonic saline in these experiments probably would not affect results. Had the technical factors been minimized the results may not have been so variable. Alternatively, and even with these limiting factors, the results did

show definite trends and could represent a test system that is just by nature a highly variable system. Other drawbacks with this test design are, the measurement of IL-6 in this experiment has been with an immunoassay. Although the ability to detect this protein immunologically is evident, it does not mean the production at elevated temperature results in a bioactive form. Cell proliferation studies can measure its retention of bioactivity and would also be a prudent experiment to perform. Additionally, molecular biology testing, particularly measuring IL-6 mRNA levels in correlation with the temperature and time parameters would also add to a more complete understanding of temperature effects on IL-6 production.

Chapter 6

CONCLUSION

Successful immune responses are dependent on proper leukocyte function. The notion that the neutrophil's role in immunity is limited to seeking out and phagocytosing bacterial pathogens is erroneous. Cytokines play a large role in mounting an effective inflammatory response and subsequent immune protection. Neutrophils produce and respond to many cytokines. The febrile response is a normal process the body uses to aid in protection against infection. Fever is mediated by endogenous pyrogens produced by leukocytes when stimulated by infection. Interleukin-6 is a well known pyrogenic cytokine and is necessary for normal immune function. It was hypothesized that elevated temperature would be a signal for repressing IL-6 production by neutrophils.

Research shows neutrophils can be induced to produce IL-6 by the endotoxin, LPS, derived from bacterial membranes. In this study it was shown that neutrophils primed with fMLP, and subsequently stimulated with LPS from *E. coli* serotype O111:B4, produced pg quantities of IL-6. IL-6 was a thermally stable protein, even at temperatures as high as 41°C. Negative controls, which were unstimulated native cells and fMLP primed only cells did not produce any detectable IL-6 after 8 hours of incubation. Although results varied from batch to batch, there were some noticeable trends. Base line levels (37° C) of IL-6 appeared only after 4 hours, and ranged from

nondetectable to 13.5 pg/ml after 8 hours. IL-6 production at 38.5° C was on average less than base line, although not in every instance. Production at 40° C was lower than base line levels in every instance. Interestingly, at 41° C there appeared to be a jump in IL-6 levels. In all parameters measured, it was much higher than base line levels. This was a departure in the trend and in contrast to the hypothesis. Feedback signals that control body temperature are very complex. They rely on several biochemical pathways that involve the neuroimmune and hormonal systems. Feedback signaling in a fever model would therefore be difficult to assess in a single cell test system. The effect of temperature in this study is more likely to be a direct effect on one or more cellular processes within the neutrophil. Temperature is known to effect enzyme function. Since CD14 signal transduction involves protein tyrosine kinase, temperature may negatively effect this or subsequent enzymatic events involved in IL-6 induction. Efficiency of production or function of nuclear transcription factors may be similarly affected. The apparent rise in IL-6 production at 41° C demonstrates an unexpected phenomenon. Perhaps there are temperature dependent factors that decrease the efficiency of repressive events of this protein production. Likewise there may be ineffective function of RNases that result in more IL-6 mRNA being translated. It is also possible that there are temperature dependent events that normally clear the stimulus (LPS) that now allow its persistence and hence more IL-6 to be produced. Certainly more testing is required to elucidate any temperature dependent mechanisms in this test system.

The lack of reproducibility among the different batches of results may be a natural phenomenon of the system. Only a large number of observations would show the degree of natural biovariability. Two identified factors could also account for the lack of reproducibility. LPS stimulation requires a CD14 receptor mediated system. Stimulation was aided by LBP supplied from autologous plasma. This may have played a role in the variability of results since it was not the same plasma used each time. Finally, reagent degradation may have occurred which would result in variable degrees of neutrophil stimulation, and hence variable levels of IL-6 production.

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Table 1

37° 1ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	0.9	7.7	9.7
Batch 2	ND	ND	ND	ND
Batch 3	ND	ND	ND	2.5

37° 10ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	2.3	8.9	13.5
Batch 2	ND	ND	ND	ND
Batch 3	ND	ND	ND	2.8

37° 100ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	1.6	7.1	13
Batch 2	ND	ND	1.2	1.0
Batch 3	ND	ND	ND	1.4

40° 1ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	1.4	3.5	7.7
Batch 2	ND	ND	ND	ND
Batch 3	ND	ND	ND	ND

40° 10ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	2.1	5.3	7.1
Batch 2	ND	ND	ND	ND
Batch 3	ND	ND	ND	ND

40° 100ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	0.9	4.4	7.1
Batch 2	ND	1.4	0.5	0.2
Batch 3	ND	ND	ND	ND

38.5° 1ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	ND	0.7	2.2
Batch 2	ND	ND	ND	ND
Batch 3	ND	ND	ND	2.7

38.5° 10ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	ND	0.7	2
Batch 2	ND	ND	0.8	ND
Batch 3	ND	ND	ND	ND

38.5° 100ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	ND	ND	2.2
Batch 2	ND	2.7	1.5	2.2
Batch 3	ND	ND	ND	1.5

IL-6 in pg/ml

41° 1ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	14.5	37.4	49.2
Batch 2	ND	ND	ND	1.7
Batch 3	ND	ND	1.5	2.1

41° 10ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	1	16.2	30.6	41.8
Batch 2	ND	1.3	5.1	4.9
Batch 3	ND	ND	1.4	4.7

41° 100 ng LPS

Time	2hr	4hr	6hr	8hr
Batch 1	ND	11.4	27.3	42.4
Batch 2	2.4	7.3	8	5.8
Batch 3	ND	ND	ND	2.1

ND = Not Detected

Figure 1

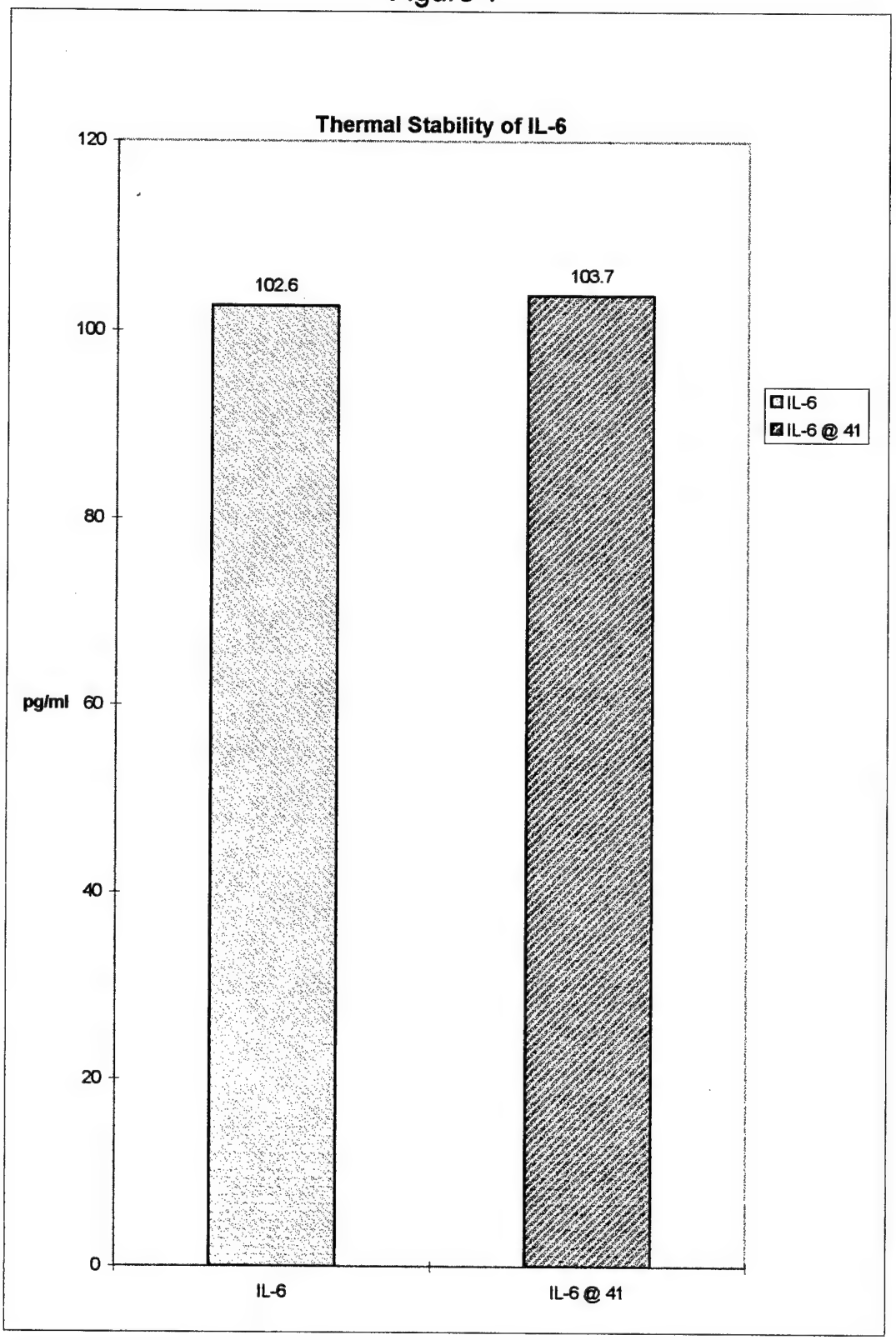
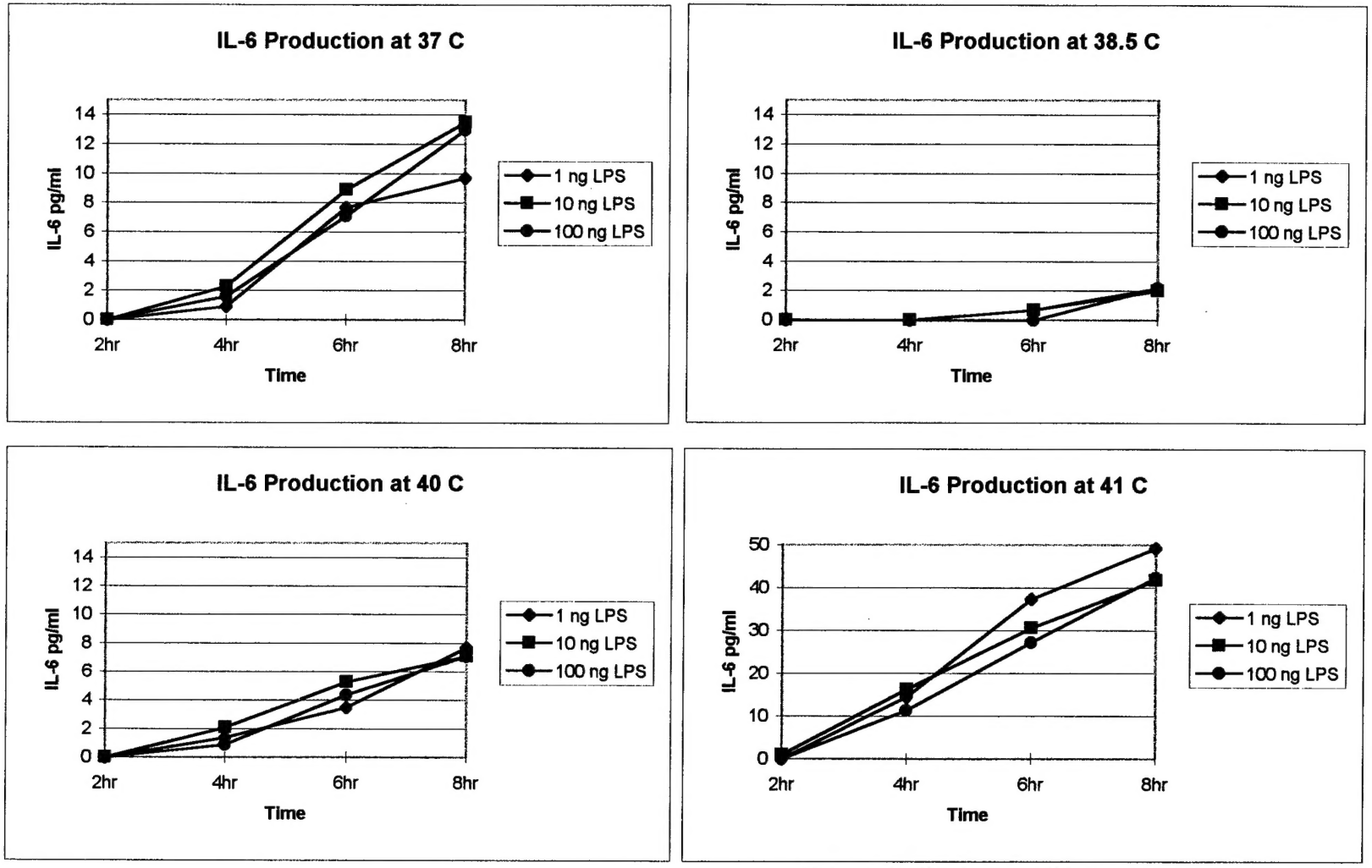

Thermal Stability of IL-6
120
100
80
60
40
20
0
pg/ml
102.6
103.7
IL-6
IL-6 @ 41
IL-6
IL-6 @ 41

Figure 2

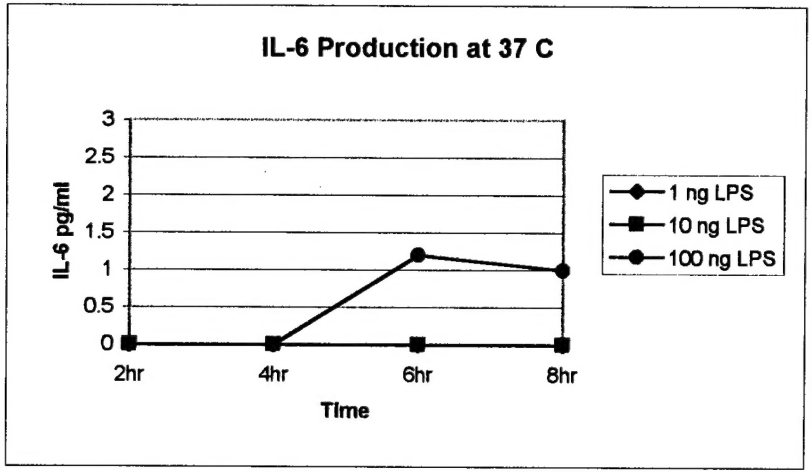
IL-6 results from batch 1

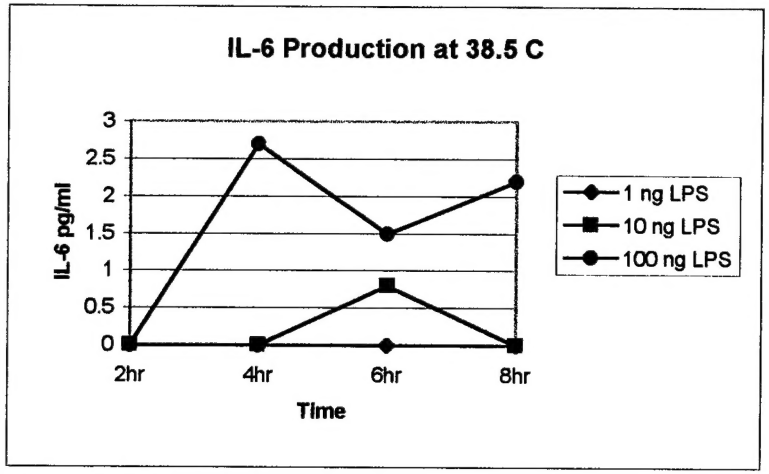


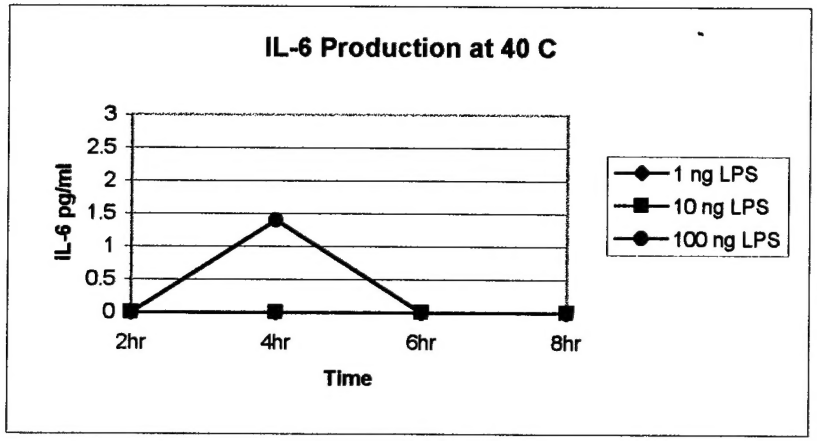
Note scale difference at this temperature

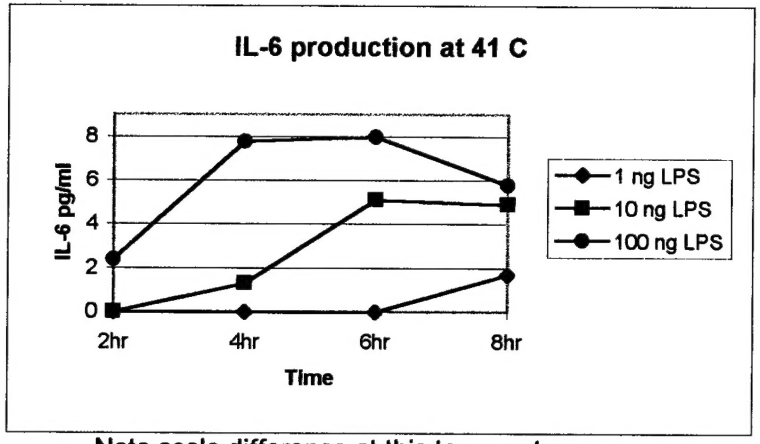
Figure 3

IL6 results from batch 2





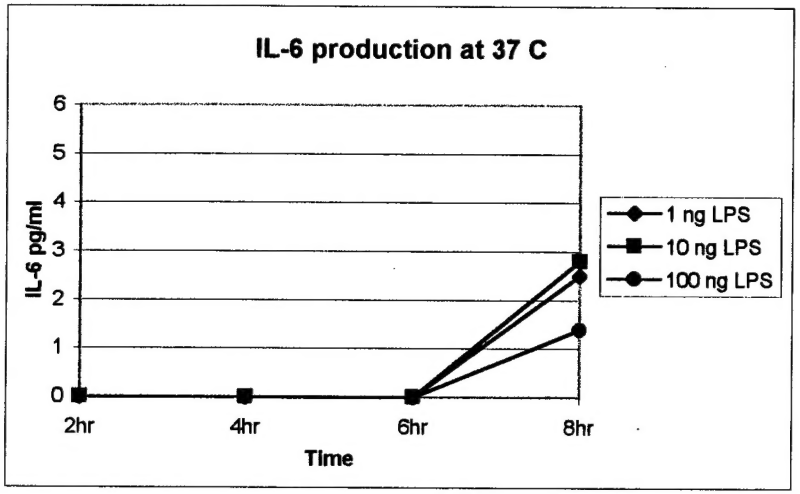


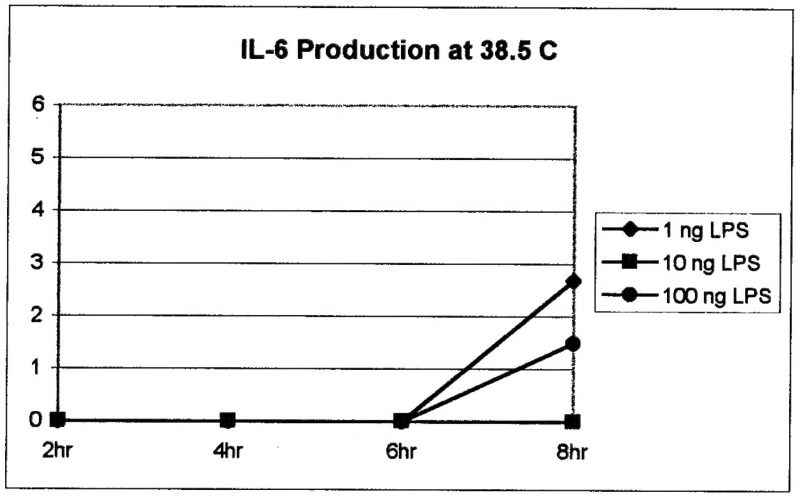


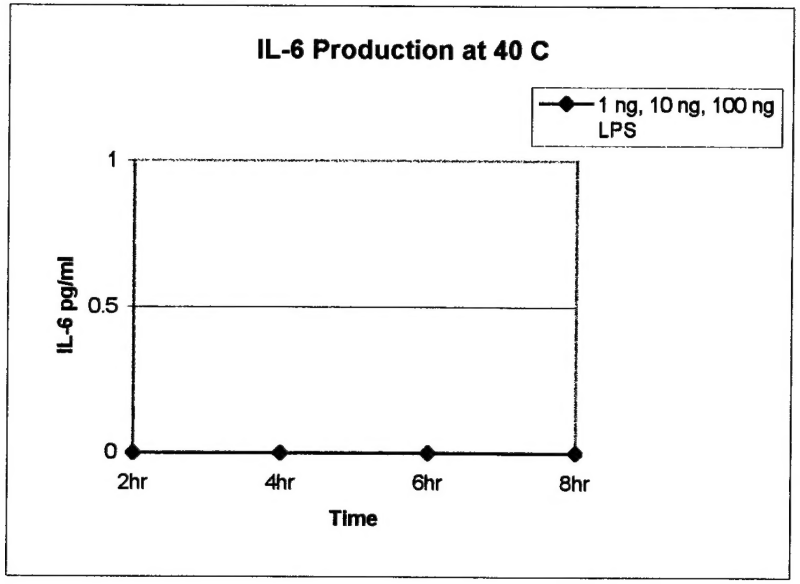
Note scale difference at this temperature

IL-6 results from batch 3

Figure 4







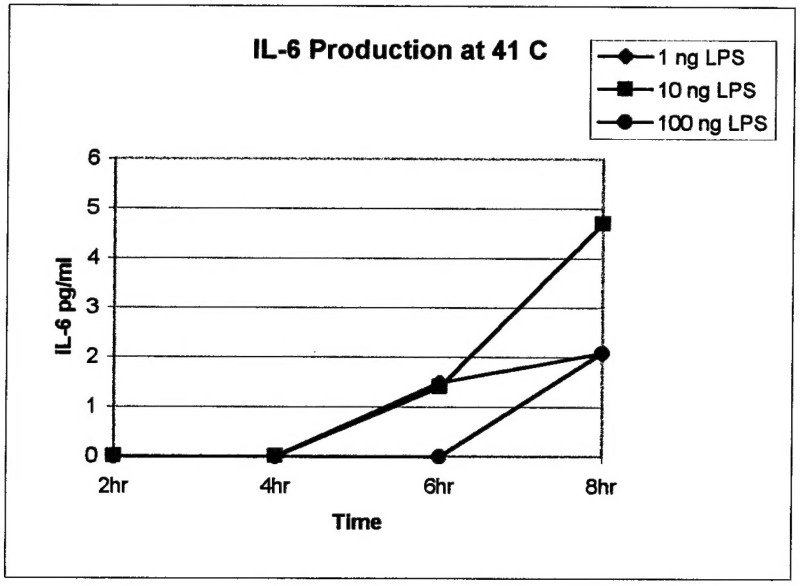
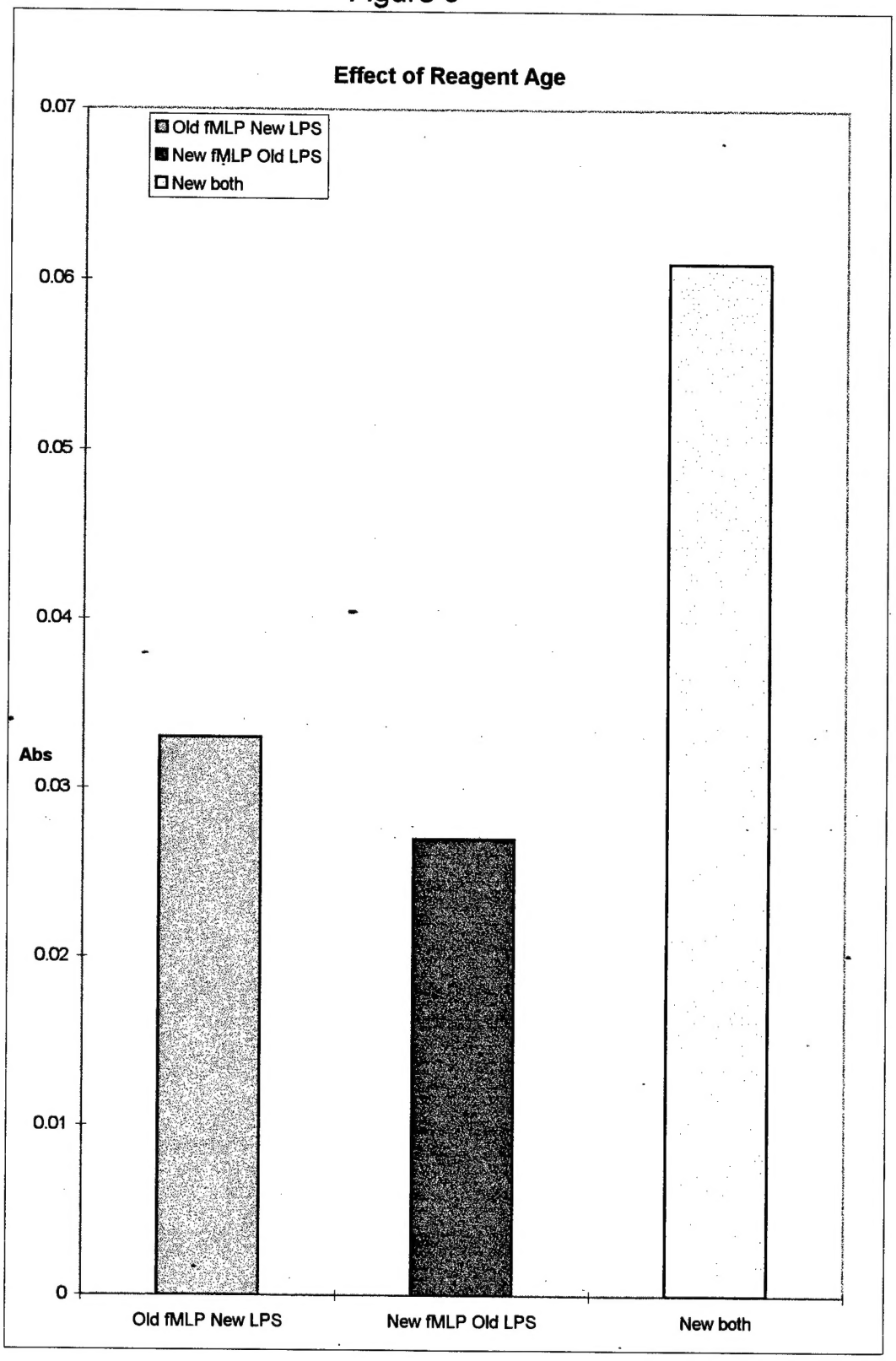


Figure 5


Effect of Reagent Age
Old fMLP New LPS
New fMLP Old LPS
New both
0.07
0.06
0.05
0.04
Abs
0.03
0.02
0.01
0
Old fMLP New LPS
New fMLP Old LPS
New both